Rapid Purification of Fumonisins B_3 and B_4 with Solid Phase Extraction Columns

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A simple method was developed to isolate fumonisins B_3 and B_4 (FB_3 and FB_4) from cultures of a novel strain of *Fusarium moniliforme* which produces FB_3 and FB_4 but does not make FB_1 or FB_2. Undiluted extract was loaded onto a 10 g NH_2 solid phase extraction cartridge, and the fumonisins were eluted with 5% acetic acid in methanol. The eluate was diluted with 1.5 vol of water and loaded onto a 10 g tC18 cartridge. The fumonisins were eluted with increasing amounts of acetonitrile in water. Complete separation was obtained between the FB_3 (277 mg) and FB_4 (62 mg) fractions. The fractions were analyzed for purity by three methods: fluorescence detection of the OPA derivatives, evaporative light-scattering detection of the underivatized fumonisins, and electrospray-MS. The fractions from the combined NH_2/tC18 method contained more than 90% fumonisins. Recovery of the fumonisins from the extracts exceeded 95%.

Keywords: *Fumonisins; Fusarium moniliforme; corn; mycotoxins; isolation*

INTRODUCTION

Fumonisin B_1 (FB_1) was originally isolated and identified as a toxic metabolite of *Fusarium moniliforme* isolated from corn (Bezuidenhout et al., 1988). FB_2, FB_3, and FB_4, which differ in their pattern of hydroxylation from FB_1, and FA_1 and FA_2, the *N*-acetylated analogs of FB_1 and FB_2, have also been identified (Cawood et al., 1991; Gelderblom et al., 1988; Plattner et al., 1992). Two fumonisins, FC_1 and FC_4, lacking the C-1 methyl group characteristic of the other fumonisins are also found in culture material (Branham and Plattner, 1993; Plattner, 1995). The predominant fumonisin found in naturally contaminated corn is FB_1, which is usually about 70% of the total fumonisins present. Purified FB_1 causes a range of toxic responses in animals including equine leucoenphalomalacia, porcine pulmonary edema, and hepatosis and nephrotoxicity in rodents (Nelson et al., 1993; Norred and Voss, 1994). FB_1 has also been associated with high incidences of human esophageal cancer in South Africa (Rheeder et al., 1992) and China (Chu and Li, 1994). Much less is known about the toxicity of the other fumonisins. When the toxicity of FB_1, FB_2, and FB_3 was compared in rats, they showed similar hepatotoxic effects and similar, although weak, cancer-initiating potential (Gelderblom et al., 1993). More work with the other fumonisins in purified form is needed to determine their toxicological significance (Norred and Voss, 1994). The use of unpurified extracts from fungal cultures to conduct toxicological studies may lead to unreliable results. Some of the effects observed may be due to other toxins that may be produced by *F. moniliforme* and not the fumonisins. *F. moniliforme* and closely related species produce several other mycotoxins and phycotoxins including moniliformin, beauvericin, fusaric acid, the fusarins, and the naphthazarine pigment complex whose toxicity has not been as well studied as the fumonisins. Even analyzing for the presence or absence of other known toxins does not eliminate the possibility the effects may be due to a yet unidentified metabolite of the mold. Because fumonisins are commonly found in grains used for animal feed and can occur in processed corn products consumed by humans (Nelson et al., 1993; Bullerman and Tsai, 1994), there has been a great deal of interest in their study.

We were interested in developing a rapid method for isolating FB_3 and FB_4 in sufficient amounts and purity to study their toxicity. FB_3 and FB_4 are difficult to isolate and purify from typical strains of *F. moniliforme* because they occur at much lower concentrations than FB_1. Atypical strains of *Fusarium proliferatum* that produced mainly FB_2 or FB_3 but little FB_1 have been isolated from equine and swine feeds (Ross et al., 1992; Nelson et al., 1994). We recently identified two strains with unusual fumonisin ratios during the screening of several hundred mating population A strains of *F. moniliforme*. One of these strains (KSU 819) accumulates high levels of FB_3 and FB_4 but virtually no FB_1 or FB_2. It also accumulates a new fumonisin, FC_4, which is the analog of FB_4 lacking the C-1 methyl group. The other strain (KSU 817) made little FB_1 or FB_3 but accumulates high levels of FB_2, FB_4, and FC_4 (Plattner, 1995; Plattner et al., 1996). Full details of the survey will be reported separately. These strains provide a convenient source of FB_2 or FB_3.

The purification of FB_3 and FB_4 from strain KSU 819 will be discussed in this paper. The method developed is rapid and requires only a minimal amount of equipment. It uses only solid phase extraction cartridges with C18- and propylamine (NH_2)-bonded phases. It can be directly scaled to different size cartridges by adjusting the volumes used proportionally to the cartridge weights. Because FB_3 and FB_4 are not readily available, this rapid method should be of interest to those wishing to test the toxicity of these fumonisins. It will provide good starting material for obtaining analytical standards by semipreparative HPLC that can be used for the identification and quantitation of these fumonisins in various grain products. It should also be useful for purifying radiolabeled fumonisins because of

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the small amounts of waste generated and the simple equipment required.

EXPERIMENTAL PROCEDURES

Fungal Strains and Culture Conditions. Strains used in this study were from the culture collection at the Fusarium Research Center at Pennsylvania State University or from the collection of Dr. John Leslie (Kansas State University). The isolates of *F. moniliforme* were grown on cracked corn using published procedures (Nelson et al., 1991).

Extraction. The corn cultures were extracted with 5 mL of CH_3CN/H_2O (1/1) for each gram of corn by soaking for 2−3 h (with mixing every 0.5 h). The resulting suspensions were filtered through Whatman #1 paper in a Buchner funnel or Whatman 2v folder filter paper in a glass funnel. For the tC18 cartridge procedure, a fresh extract that contained about 2.5 mg/mL FB_3 was prepared from a single 50 g flask of corn that produced a very high level of FB_3. The extracts from several other flasks were combined to give 3.5 L of pooled extract that contained about 1.1 mg/mL FB_3.

HPLC. Previously purified FB_3 was used as the standard against which to measure the other components of the fractions. None of the other components were available in sufficient quantity of known purity to serve as standards. The responses are based on peak areas. Three different systems were used to perform the analyses. The OPA derivatives (Shephard et al., 1990) were analyzed on a Pecosphere 3 × 3C C18 column (0258−0164, Perkin Elmer Corp., Norwalk, CA) using a fluorescence detector (excitation 334 nm, emission 440 nm). The flow rate was 1.0 mL/min. Solvent A was 60/40 methanol (MeOH)/0.1 M NaH_2PO_4 adjusted to pH 3.35 with H_3PO_4 after mixing. Solvent B was 80/20 MeOH/0.1 M NaH_2PO_4 adjusted to pH 3.35. The gradient was held at 25/75 A/B for 1 min, and then a linear gradient was run to 0/100 A/B over 15 min and held at 100% B for 5 min (t_R: FB_1 9.9 min, FB_2 15.3, FB_3 14.8, FB_4 20.1).

The underivatized fumonisins were analyzed using an evaporative light-scattering detector (Varex Mark III ELSD detector, Alltech Associates, Deerfield, IL). Initial qualitative analyses were performed using a Pecosphere 3 × 3C C18 column as for the OPA derivative but the following solvent system. The flow rate was 0.5 mL/min. Solvent A was 700/300/1 acetonitrile (CH_3CN)/H_2O/TFA (trifluoroacetic acid). Solvent B was 200/800/1 CH_3CN/H_2O/TFA. The gradient was held at 20/80 A/B for 1 min, and then a linear gradient was run to 50/50 A/B over 15 min (30−45% CH_3CN) and held at 50/50 A/B for 5 min (t_R: FB_3 10.8 min, FA_3 13.6, FB_4 17.3, FA_4 20.8).

Quantitative analyses with the ELSD (tube temperature 110 °C, gas flow 2.75 slpm) were performed using an Inertsil ODS-3 15 cm × 3.0 mm i.d. column (0396-150 × 030, MetaChem Technologies, Torrance, CA). The flow rate was 0.5 mL/min. Solvent A was 1000/1 MeOH/acetic acid (HOAc). Solvent B was 500/500/1 MeOH/H_2O/HOAc. The gradient was held at 20/80 A/B for 1 min, and then a linear gradient was run to 70/30 A/B over 15 min (60−85% MeOH) and held at 70/30 A/B for 5 min (t_R: FB_3 8.2 min, FA_3 17.8, FB_4 13.5, FA_4 22.1, FC_4 13.2).

For the third system, the HPLC was coupled to a Finnigan-MAT TSQ 700 mass spectrometer via the Finnigan electrospray interface (ESI) (Finnigan-MAT, San Jose, CA). A YMC Basic 25 cm × 2 mm i.d.D column (BA99S052502PFK, YMC, Inc., Wilmington, NC) was used for the ESI measurements. The flow rate was 0.4 mL/min. The solvents and gradient program were the same as those used with the Inertsil ODS-3 column described above (t_R: FB_3 6.4 min, FA_3 15.2, FC_3 5.5, FB_4 13.2, FA_4 18.4, FC_4 13.1). The total HPLC eluent was introduced into the detector. Mass spectra were obtained by scanning from m/z 350/950 in 1.5 s.

Preparation of Analytical Samples. As originally dried, the fractions from the separation procedures given below were obtained as viscous residues. Because the residues entrap solvent and give inconsistent dry weights, the residues were dissolved in MeOH, a large portion of the MeOH solution was evaporated under a N_2 stream at room temperature, and the residues were dissolved in a small volume of CH_3CN/H_2O (50/50), freeze-dried to an amorphous powder, and weighed. The powders weighed between 74% and 91% of the starting weight of the residues. The dry weights of the powders were used to calculate the actual concentrations of the analytical samples. Aliquots of the remaining MeOH solutions were diluted with 50/50 CH_3CN/H_2O to the appropriate concentration for each method.

tC18 Cartridge Procedure. A tC18 cartridge containing 10 g of packing material (Sep-Pak Vac 35 cc (10 g) tC18 cartridge, WAT043350, Waters Corp., Milford, MA) was conditioned with 100 mL of MeOH and then 100 mL of 10/90 CH_3CN/H_2O; 80 mL of fresh extract from the high-FB_3-producing culture (about 2.5 mg/mL FB_3 in 50/50 CH_3CN/H_2O) was diluted to 400 mL with H_2O and loaded onto the cartridge. The cartridge was eluted with 100 mL aliquots of 20/80, 30/70, 40/60, 50/50, 60/40, and 100/0 CH_3CN/H_2O. The 30/70, 40/60, and 50/50 aliquots contained the compounds of interest as determined by HPLC and were freeze-dried using a VirTis Vacu-spin (The VirTis Corp., Gardener, NY).

NH_2 Cartridge Procedure. A 2 g NH_2 cartridge (Sep-Pak Vac 12 cc (2 g) NH_2 cartridge, WAT054650) was conditioned with 50 mL aliquots of MeOH and then 50/50 CH_3CN/H_2O; 50 mL of the pooled extract (about 1.1 mg/mL FB_3 in 50/50 CH_3CN/H_2O) was loaded onto the cartridge which was eluted with 50 mL aliquots of 50/50 MeOH/H_2O, MeOH, and 5% HOAc in MeOH. The fumonisins are in the 5% HOAc/MeOH fraction. This was either freeze-dried or evaporated under a N_2 stream.

Combined NH_2/tC18 Cartridge Procedure. Pooled extract (250 mL) was loaded onto a 10 g NH_2 cartridge (Sep-Pak Vac 35 cc (10 g) NH_2 cartridge, WAT054740) that had been conditioned and was subsequently eluted with 250 mL aliquots as described above. The 250 mL 5% HOAc/MeOH fraction was diluted to approximately 40% MeOH by adding 375 mL of H_2O. A 10 g tC18 cartridge was conditioned with 100 mL aliquots of MeOH and 40/60 MeOH/H_2O. The diluted sample was loaded onto the tC18 cartridge. The presence of HOAc required a slight modification of the procedure used for the tC18 cartridge alone. The cartridge was eluted with 100 mL aliquots of 15/85, 25/75, 35/65, a second 35/65, 45/55, and 55/45 CH_3CN/H_2O. The combined 35/65 aliquots contained FB_3, and the 45/55 fraction contained FB_4. Alternatively, the excess HOAc can be removed by washing the tC18 cartridge with 100 mL of 40/60 MeOH/H_2O after the fumonisins have been loaded. Air is then briefly pulled through the cartridge to remove the void volume but not drying the packing material. The combined fumonisins can then be eluted with MeOH.

RESULTS AND DISCUSSION

Because only FB_3 was available to prepare a weighed standard and because some fractions contained several fumonisins in lower amounts that were not detected by all analytical methods, each of the fractions was analyzed by three different methods to obtain a more complete picture of its composition. Each of the analytical methods (the OPA derivative, ELSD, or ESI) has advantages and disadvantages. The fluorescence detection of the OPA derivative is a widely used method, has a linear response, and gives very good analytical results when used with standard FB_1, FB_2, and FB_3. The formation of the OPA derivative requires a free amine group so this method is unable to measure the *N*-acetylated analogs. Also, the OPA derivative is unstable under the acidic conditions used for HPLC, causing different responses to be observed for FB_1, FB_2, and FB_3. The response decreases as the retention time increases. Therefore, the response observed for FB_4 will be less than that for the same amount of FB_3 because of its longer retention time. Because the amount of FB_4 was estimated by assuming that the response factors for FB_3 and FB_4 were the same, the amount of FB_4 reported in Table 1 as by OPA will be less than the actual amount.

Table 1. Comparison of the Fumonisin Composition of the Fractions from the tC18 and NH_2 Cartridges As Measured by Evaporative Light-Scattering Detection (ELSD), Electrospray-MS (ESI), or Fluorescence Detection of the *o*-Phthalaldehyde Derivative (OPA)

method	wt (mg)[a]	fumonisin	% by ELSD	% by ESI	% by OPA[b]
tC18 cartridge (10 g)	175	FB_3	77	69	78
		FA_3[c]		tr	
		FC_3		1	
		total[d]	77	71	78
	60	FB_4	56	53	na
		FA_4	6	2	
		FC_4	6	8	
		total[d]	68	63	
NH_2 cartridge (2 g)	66	FB_3	53	72	53
		FA_3	8	4	
		FC_3		1	
		FB_4	11	15	9
		FA_4		tr	
		FC_4		2	
		total[d]	72	95	62
combined NH_2/tC18 cartridges	277	FB_3	80	76	83
		FA_3	16	7	
		FC_3		2	
		total[d]	96	85	83
	62	FB_4	85	77	61
		FA_4	8	3	
		FC_4	15	13	
		total[d]	108	92	61

[a] Total dry weight of each fraction. [b] FA_3 and FA_4 do not react with OPA reagent and cannot be detected by this method. [c] FA_3 elutes in the fraction before this fraction when the tC18 cartridges are used alone. [d] Percentage of the dry weight of each fraction that can be attributed to the fumonisins in that fraction as determined by the ELSD, ESI or OPA analytical method.

Detection and quantitation of underivatized fumonisins by an evaporative light-scattering detector (ELSD) has been reported (Wilkes et al., 1995; Plattner, 1995; Plattner et al., 1996). Because the response of the ELSD is only dependent upon the mass of the eluate, all fumonisin analogs should give the same response as FB_3 as long as the peak shape remains the same over the entire chromatogram. The HPLC method used for the quantitative analysis meets this condition. The response curve was determined each day from single injections of 200, 500, 1000, 1500, and 2000 ng of FB_3. A ln−ln plot of the response against the amount of standard was linear with coefficients of determination of $r^2 = 0.998$ and 1.000 on 2 consecutive days. The detection limit by ELSD (about 10 ng) is not as low as that of the OPA derivative with fluorescence detection (about 0.1 ng) but is entirely adequate for the analysis of the amounts of fumonisins found in *Fusarium* cultures.

Electrospray-MS is an ideal technique to detect and measure fumonisins. The protonated molecule (*m/z* 706) of FB_3 is the base peak in the mass spectrum, and little fragmentation is observed. This is also true for FB_4, FC_3, and FC_4 with base peaks at *m/z* 690, 692, and 676, respectively. Quantitation in ESI was based on the area of the protonated molecular ion (*m/z* 706) of the FB_3 standard. Three injections each of 100, 200, 300, 400, and 500 ng of FB_3 were made over the several days the analysis was performed. The values for each amount were averaged and plotted to obtain a standard curve. For 300 ng and below, a linear response with a coefficient of determination of $r^2 = 1.000$ was obtained which was good down to 10 ng. The linear response curve for FB_3 was used to measure the amounts of FA_3, FA_4, FC_3, and FC_4. Above 300 ng, the response became nonlinear, and the standard curve was best described by a second-order polynomial with a coefficient of determination of $r^2 = 0.997$. The second-order equation was used to calculate the amounts of FB_3 and FB_4. Quantitation of FC_3 and FC_4 is based on the areas of their respective protonated molecular ions compared to that of the FB_3 standard. They are similar enough to FB_3 that they should have similar ionization efficiencies and give reasonably accurate measurements. Under our experimental conditions the sodiated molecular ions of FA_3 and FA_4 at *m/z* 770 and 754, respectively, were the base peaks. For FA_3 and FA_4, the sum of the areas of protonated and sodiated molecular ions was compared to the area of the protonated molecular ion of the FB_3 standard. Because the ionization efficiencies of the amides are less than those of the free amines, they will be underestimated by ESI. Another advantage of electrospray-MS is that while FC_3 was not resolved from FB_3 with the HPLC conditions used for ELSD or ESI and the measured value for FB_3 by ELSD includes a small amount of FC_3, by monitoring the protonated molecular ion by ESI, FC_3 could be detected and measured.

The results from the quantitative analyses of the various fractions are shown in Table 1. Purification of the fumonisins was by means of reversed-phase chromatography on tC18 cartridges. When 16 mL of the fresh extract was separated on a 2 g tC18 cartridge, three fractions contained compounds of interest. FA_3 was the main component of the 30% CH_3CN fraction which also contained FB_3. Most of the FB_3 eluted with 40% CH_3CN. FB_4 eluted in the 50% CH_3CN fraction. The procedure was scaled up to 10 g cartridges to determine the amount and purity of FB_3 and FB_4 that could be obtained for toxicity studies. The 20 mL aliquot size used for the 2 g cartridges was scaled to 100 mL for use with 10 g cartridges, and the same separations were obtained. The results are shown in Table 1. The FB_3 fraction (175 mg) eluted with 40% CH_3CN and contained 69−78% FB_3 depending on which method of analysis was used. Thus the three methods agree reasonably well given that each has approximately a 5% deviation. This fraction also contained about 1% FC_3 (the analog lacking the C-1 carbon) and a trace of FA_3. The FB_4 fraction (60 mg) contained 53−56% FB_4, 2−6% FA_4, and 6−8% FC_4. The amount of FA_4 is probably closer to 6% because ESI underestimates the amides. The percentage of FC_4 in the FB_4 fraction is much higher than the percentage of FC_3 in the FB_3 fraction. FC_4 occurred in similar though larger amounts than FC_3 in all the samples analyzed even though the amount of FB_3 is about 5 times greater than that of FB_4. In all cultures examined to date, the level of FC_4, while always quite low, has also been observed to occur at much greater levels than those of FC_1 (Plattner et al., 1996). The tC18 procedure gives a good separation between the FB_3 and FB_4 fractions and fractions that are more than 70% fumonisins.

The identification of FC_3, FA_4, and FC_4 in the fractions was based on the following criteria. FC_3 was tentatively identified based on electrospray-MS and its chromatographic behavior. It elutes shortly before FB_3 just as FC_1 and FC_4 elute shortly before FB_1 and FB_4, respectively. The strong signal at *m/z* 692 in the positive ion mode is 14 Da less than that observed for FB_3, as would be expected for the loss of the C-1 methyl group. The characterization of FA_3 and the tentative identification of FA_4 has been previously described

(Plattner et al., 1996). Work is currently underway to purify and completely identify these minor fumonisin analogs. FC_4 has been previously identified in cultures of strain KSU 819 (Plattner, 1995).

Purification of the fumonisins was also performed by means of weak anion-exchange chromatography using NH_2 cartridges. After conditioning the cartridge, the 50/50 CH_3CN/H_2O extract can be loaded onto the cartridge without dilution. After washing the cartridge, the fumonisins are eluted with 5% HOAc/MeOH. The results from using a 2 g NH_2 cartridge are shown in Table 1. The percentages of FB_3 and FB_4 as measured by ELSD and OPA are in close agreement. The values measured by ESI are higher than those measured by ELSD in this fraction, while in the other fractions they are similar or lower. This is probably due to the way the sample was prepared. Initially, the sample was dried under a nitrogen stream which gives a viscous residue that retains HOAc. It was dissolved in MeOH, the analytical samples were removed, and the bulk of the remainder was freeze-dried to an amorphous powder that had only 74% of the initial weight. The extra acid probably caused the increased response seen by ESI. Electrospray sensitivity is greatly affected by pH and ion strength of the eluting solvent (Plattner et al., 1996). The NH_2 cartridge separates the fumonisins as a group. The extracts do not need to be diluted before being loaded onto the cartridge to prevent them from being lost. This saves time compared to the tC18 procedure where a large volume of diluted extract must be loaded onto the cartridge to prevent the fumonisins from passing through the cartridge without being retained. The NH_2 cartridge is also very good at removing pigments from the fumonisin fraction. The pigments either pass through when the extract is loaded onto the cartridge or are more strongly retained than the fumonisins and are not eluted with them. The NH_2 cartridge procedure can easily and rapidly yield a fumonisin fraction that is 70% fumonisins starting with extracts containing 0.1% FB_3.

Finally, the NH_2 and tC18 procedures were combined to take advantage of both weak anion-exchange and reversed-phase chromatography. The NH_2 cartridge procedure was performed as described above. The 5% HOAc/MeOH fraction was diluted with water to a 40% organic solution and loaded onto a preconditioned tC18 cartridge. The presence of the acid reduced the amount of CH_3CN required to elute the fumonisins and caused FB_3 and FA_3 to elute in the same fraction. The FB_3 fraction (FB_3, FA_3, and FC_3) eluted with 35% CH_3CN. The majority of the FB_3 eluted in the first aliquot of 35% CH_3CN, but a second aliquot of 35% CH_3CN was required to keep FB_3 from being eluted in the FB_4 fraction. Because there was no FB_4 in the second aliquot of 35% CH_3CN, it was combined with the first aliquot. In some cases, with other samples, a small amount of FB_4 eluted in the second aliquot. The FB_4 fraction (FB_4, FA_4, and FC_4) elutes with 45% CH_3CN. The amount of material that can be loaded onto the NH_2 cartridge is the limiting factor that determines the amount of fumonisins that can be obtained by this procedure. In no instances, when the cartridges were the same size, did the eluent from the NH_2 cartridge cause the tC18 cartridge to be overloaded. If the amount of extract that could be loaded onto a 360 mg NH_2 cartridge was determined, then 5 and 25 times as much could be loaded on 2 and 10 g cartridges, respectively, and successful separations were obtained. The results using a 10 g NH_2 and a 10 g tC18 cartridge are shown in Table 1. Because the OPA method does not measure FA_3 or FA_4 and ESI underestimates them, the FB_3 and FB_4 fractions were at least 90% fumonisins and may be greater than 95% fumonisins. A few percent of various methyl esters of FB_3 or FB_4 arising from their exposure to MeOH were also present in the fractions. The recovery of the fumonisins from the extracts by the combined NH_2/tC18 procedure was greater than 95%.

By using the combined NH_2/tC18 cartridge procedure and applying it to extracts from *F. moniliforme* strain KSU 819, FB_3 and FB_4 fractions of high purity were obtained. Starting with extract from strain KSU 817, it should be possible to isolate FB_2 and FB_4 fractions of similar purity. The method is easy, requires only simple equipment to carry out, and can be completed in a few hours. Sufficient FB_2, FB_3, and FB_4 should be obtained to establish feeding levels for toxicity studies in small animals and to obtain more information on their relative toxicity and their toxicity compared to that of FB_1.

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